PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/00 | A2

(11) International Publication Number: **WO 99/46368**

(43) International Publication Date: 16 September 1999 (16.09.99)

(21) International Application Number: PCT/US99/05722

(22) International Filing Date: 12 March 1999 (12.03.99)

(30) Priority Data:
09/039,066 13 March 1998 (13.03.98) US

(71) Applicant (*for all designated States except US*): BIOMARIN PHARMACEUTICALS [US/US]; Suite 210, 371 Bel Marin Keys Boulevard, Novato, CA 94949 (US).

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): KLOCK, John, C. [US/US]; 345 Willis Road, Nicasio, CA 94946 (US).

(74) Agent: HALLUIN, Albert, P.; Howrey & Simon, Box 34, 1299 Pennsylvania Avenue, N.W., Washington, DC 20004 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: CARBOHYDRATE-MODIFYING ENZYMES FOR BURN AND WOUND DEBRIDEMENT AND METHODS FOR TREATMENT

(57) Abstract

The present invention features a method for treating wounds comprising the step of administering an effective amount of a carbohydrate-active enzyme. In another aspect, the present invention features pharmaceutical compositions for treating wounds. The invention also features cDNA sequences for carbohydrate-active enzymes and cDNA expression systems for using various DNA sequences to produce mammalian carbohydrate-active enzymes for a variety of uses. The invention further includes recombinant plasmids comprising the cDNA of mammalian carbohydrate-active enzymes. The invention also includes transformed cells comprising a heterologous DNA sequence that encodes for a carbohydrate-active enzyme or a biologically active fragment or mutant thereof. Preferred carbohydrate-active enzymes according to the present invention include chondroitinases and hyaluronidases.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

CARBOHYDRATE-MODIFYING ENZYMES FOR BURN AND WOUND DEBRIDEMENT AND METHODS FOR TREATMENT

FIELD OF THE INVENTION

The present invention is in the field of carbohydrate chemistry and biochemistry. In particular, the invention provides carbohydrate-cleaving enzymes having broad-specificity and methods for debriding burns and other wounds using the same.

BACKGROUND OF THE INVENTION

Carbohydrates play a number of important roles in the functioning of living organisms. In addition to their metabolic roles, carbohydrates are structural components of the human body, being covalently attached to numerous other entities such as proteins (glycoproteins) and lipids (glycolipids) and are called collectively "glycoconjugates". For example, the human skin makeup consists of collagen proteins, sugar-containing glycolipids, and a sugar-containing protein matrix called the proteoglycan matrix. The carbohydrate portion of this proteoglycan matrix imparts important properties to the skin structure such as stability and resistance against heat, acids, protease enzymes of inflammation and as a storage location for growth factors, salts, metals and water.

Burns and other wounds are characterized by abnormalities in the microarchitecture of the skin tissue leading to a reduction in permeability and strength and an increased susceptibility to fluid loss and infection. More than 2 million Americans suffer serious burns every year and approximately 150,000 of these persons require surgical treatment and skin grafting for their burns.

The current treatment of severely burned patients has dramatically reduced the incidence of death from shock, sepsis and hypermetabolism. As a result the major issues facing burn specialists are finding adequate means to re-cover the burned area and in providing chronic rehabilitation from skin contracture (Nguyen *et al., Annals of Surgery* 223:14-25 (1996)). Part of the strategy in burn wound care is to surgically remove burned skin down to the subcutaneous tissue layer and replace it with autologous, allogeneic or xenogeneic skin. Traditional burn wound management involved applying local antibiotics to burned skin and waiting until the burn eschar separated in 3-5 weeks due to liquefaction of necrotic burn tissue by proteolytic enzymes released from proliferating pathogens within the

wound. In an effort to speed up release of necrotic tissues and reduce the need for surgical debridement, the topical application of microbial proteolytic enzymes have been used. These include non-specific proteases (Durham *et al. J Burn Care Rehabil* 14:544-551, 1993 and Sinclair *et al., Australas J Dermatol* 35:35-41 (1994)) and collagenase (Hansbrough *et al., J Burn Care Rehabil* 16:241-247 (1995)). Wound sites treated with these enzymes often clean in less time and heal faster, but the treatment can be associated with pain and bleeding of the wound bed.

Identifying the components of the wound environment has become a major goal of wound healing research. One approach to study the wound environment has been through analysis of wound fluid (Young *et al., J. of Invest Dermatol.* 103:660-664 (1994)). A family of proteinases termed metalloproteinases including collagenase, gelatinase and stromelysin has been identified and found to be responsible for degradation of extracellular matrix components (Woessner *et al., FASEB J.* 5:2145-2154 (1991)). However, these enzymes are regulated by tissue inhibitory metalloproteinases exerting an inhibitory role in remodeling of the extracellular matrix especially in chronic wounds (Lafuma *et al., J. Invest Dermatol.* 102:945-950 (1994)). A recent controlled, randomized prospective trial has shown the efficacy of enzymatic debridement of partial-thickness burn wounds in limited areas with collagenase versus the topical antimicrobial silver sulfadiazine (Hansbrough *et al., J. of Burn Care and Rehabilitation* 16:241-7 (1995)). However, deep-partial and full-thickness burn wounds still provide a challenge in terms of eschar removal. The management of these burn wounds initially requires mechanical debridement of the burned skin and application of topical antibiotics. Eventually these wounds will require surgical excision and complete closure with autograft or placement of a temporary coverage using allograft. The latter will allow for neovascularization and prevent wound infection until autografting can be performed.

Recent advances in carbohydrate analysis have resulted in new interest in the role of complex carbohydrates in different biological systems (Hu *et al., J. of Chromatography.* 705:89-103 (1995)). The development of new techniques such as FACE® has provided a simple and inexpensive method for analysis of complex carbohydrates (Starr *et al., J. of Chromatography* 720:295-321 (1996)). Specific enzymes (carbohydrate modifying enzymes) for carbohydrate degradation have been applied for functional and structural studies of glycan

moieties, but almost no attention has been paid to the biological significance of these enzymes as well (Hansbrough *et al.*, *supra*). Limited information is available in the field of wound healing regarding the proteoglycans and glycosaminoglycans in the extracellular matrix. Proteoglycans are extracellular matrix, or membrane-associated macromolecules, containing 95% polysaccharide by weight and 5% protein by weight (Boehringer-Mannheim, Biochemical Products Division, Indianapolis, IN. Technical Bulletin "Biochemicals for Glycoprotein and Carbohydrate Research", pp. 124-134). The proteoglycan has a backbone of hyaluronic acid with covalently attached subunits. These subunits have a protein core and charged polysaccharides (glycosaminoglycans, GAG's) attached to the core ("Biochemicals for Glycoprotein and Carbohydrate Research", pp. 124-134, *supra*). The GAG chains are linear polymers consisting of a disaccharide repeating unit usually of a hexuronic acid (or galactose in keratan sulfate) linked to an N-acetyl-hexasamine sulfate or N-acetyl glucosamine in hyaluronic acid ("Biochemicals for Glycoprotein and Carbohydrate Research", pp. 124-134, *supra*). In the skin, their rapid turnover during development implies a dynamic role in the morphogenic processes which take place in the skin (Goldsmith, L. *Proteoglycans: Modular Macromolecules of the Extracellular Matrix. Physiology, Biochemistry, and Molecular Biology of the Skin*, 1991, Vol. I; 558-575).

The proteoglycan composition of the skin is complex, and that of the eschar is yet to be determined. Great interest exists in determining the proteoglycan composition of the extracellular matrix in eschar and normal human skin utilizing a variety of carbohydrate-modifying enzymes. These enzymes also called glycosidases are a set of enzymes found in lysosomes and used in cleavage of complex carbohydrates into simple units (Aronson Jr *et al.*, *The Faseb Journal*, 3(14):2615-22 (Dec. 1989)). These enzymes can be classified as exoglycosidases or endoglycosidases. Exoglycosidases work by enzymatic release of a particular glycosyl unit only when it is present at the nonreducing end of an oligosaccharide chain. Endoglycosidases act by catalyzing internal chain cleavage and release oligosaccharides as products (Aronson Jr *et al.*, *supra*). They also catalyze the cleavage of the linkage between the proximal saccharides and core proteins of the parent glycoconjugates (Suzuki *et al.*, *Glycobiology* 4(6):777-789 (1994)).

It has recently been determined that normal human skin has approximately 10% of its dry weight in the form of non-protein glycosaminoglycans. Glycosaminoglycans are sugar

chains consisting of repeating polymers of acidic polysaccharides. These materials are composed of building blocks of the following sugars in various combinations: galactose, glucose, N-acetylglucosamine, N-acetylgalactosamine, glucuronic acid, galacturonic acid and iduronic acid. In addition these sugar units may be variably linked α or β at their anomeric carbons and (1-3) or (1-4) to their ring carbons through an O-glycosidic bond. Finally they may be variably substituted with sulfates at their 2,3,4 or 6 carbons. Depending on the precise repeating disaccharide structure and location of sulfates, human connective tissue glycosaminoglycans are commonly classified as hyaluronates, chondroitin sulfates, dermatan sulfates, heparan sulfates, heparin sulfates and keratan sulfates (Collins PM, Carbohydrates, London, Chapman Hall, (1987)). Glycosaminoglycans are carbohydrates which are integrally related to collagen and comprise a portion of proteoglycans found in connective tissue. Normal skin is 70% hyaluronate, 20% dermatan and 10% keratan . Burn skin is thought to have only hyaluronate and dermatan.

Because of the high concentrations of glycosaminoglycans in skin, in burn patients, enzymes that degrade glycosaminoglycans (GAGs) might be useful adjuncts to burn wound debridement.

BRIEF SUMMARY OF THE INVENTION

In a first aspect, the present invention features a method for treating wounds comprising the step of administering an effective amount of a carbohydrate-active enzyme. Preferred carbohydrate-active enzymes of the present invention include but are not limited to chondroitinases and hyaluronidases. The method is particularly applicable to mucocutaneous wounds such as burn wounds but also is effective for other types of mucocutaneous wounds such as those caused by trauma including surgical wounds.

In another aspect, the present invention features pharmaceutical compositions for treating wounds. The pharmaceutical compositions in accordance with the present invention comprise an effective amount of at least one carbohydrate-active enzyme. Preferred carbohydrate-active enzymes of the present compositions include but are not limited to chondroitinases and hyaluronidases. The compositions are particularly applicable to treating burn wounds but are also effective for other types of wounds such as those cause by trauma including surgical wounds. In some preferred embodiments, the composition comprising a

carbohydrate-active enzyme is administered by topical, cutaneous or mucosal administration. In other preferred embodiments, the carbohydrate-active enzyme may be administered by cellular transformation vectors containing nucleic acid sequences encoding the carbohydrate-active enzymes.

In further aspect, the invention features cDNA sequences for carbohydrate-active enzymes and cDNA expression systems for using various DNA sequences to produce mammalian carbohydrate-active enzymes for a variety of uses. The invention further includes recombinant plasmids comprising the cDNA of mammalian carbohydrate-active enzymes. The invention also includes transformed cells comprising a heterologous DNA sequence that encodes for a carbohydrate-active enzyme or a biologically active fragment or mutant thereof.

In yet another aspect, the present invention features methods for producing a carbohydrate-active enzyme or a biologically active fragment or mutant thereof comprising the steps of culturing a transformed cell comprising a DNA sequence encoding for a carbohydrate-active enzyme or a biologically active fragment or mutant thereof in a suitable nutrient medium and isolating the carbohydrate-active enzyme or a biologically active fragment or mutant thereof.

In another aspect, the present invention features a kit for treating wounds comprising a pharmaceutical composition having at least one carbohydrate-active enzyme therein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 represents the monosaccharide analysis of normal skin. Specifically, the amounts of GalNAc, mannose, glucose, galactose, GlcNAc, and unknown are provided for whole skin and the epidermis.

Figure 2 represents the monosaccharide analysis of eschar from full-thickness burns. Specifically, the amounts of GalNAc, mannose, glucose, galactose, GlcNAc, and unknown are provided for whole eschar, mid section eschar and the epidermis.

Figure 3 represents the glycosaminoglycan composition of normal skin. Relatively large amounts of hyaluronic acid are present with lesser amounts of dermatan sulfate and keratan sulfate.

Figure 4 represents the glycosaminoglycan composition of the outer layer of normal skin. Relatively large amounts of hyaluronic acid are present with lesser amounts of dermatan sulfate and keratan sulfate.

Figure 5 represents the glycosaminoglycan composition of the inner layer of normal skin. There are similar amounts of hyaluronic acid and dermatan sulfate with smaller amounts of CSA (chondroitin sulfate A).

Figure 6 represents the glycosaminoglycan composition of total eschar. Hyaluronic acid comprises the majority of total glyocsaminoglycans with lesser amounts of dermatan sulfate.

Figure 7 demonstrates that the purified enzyme Chondroitinase ABC catalyzes the eliminative cleavage of N-acetylhexosaminide linkages in chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, chondroitin, and hyaluronic acid, yielding mainly disaccharides with Δ4-hexuronate at the non-reducing ends. The enzyme does not substantially act on keratan sulfate, heparin, and heparan sulfate. Lane 1 represents a quantification control. Lane 2 has no enzyme control. Lane 3 demonstrates that Chondroitinase ABC digests GAGs present in skin. Lane 4 represents the disaccharide standards that chondroitinase ABC effectively digests.

Figure 8 represents the effect of decreasing the time to wound healing. Wound closure of wounds treated with collagenase and chondroitinase ABC occurred in 25 and 14 days respectively.

Figure 9 represents that at the dose tested (1400 units/gm vehicle), optimal hydrolysis of eschar from the surface of partial-thickness burns is evident after two successive treatments with hyaluronidase.

Figure 10 represents that incomplete hydrolysis and nominal effects on eschar are observed after two treatments (48 hours of hydrolysis) within wounds receiving phosphate buffered saline.

Figure 11 represents that at the dose tested (1400 units/gm vehicle), optimal hydrolysis of eschar from the surface of partial-thickness burns is evident after two successive treatments with chondroitinase ABC.

Figure 12 represents the nucleotide sequence for the naturally-occurring gene encoding chondroitinase B obtained from *Falciparum heparinum.*

Figure 13 represents the nucleotide sequence for the naturally-occurring gene encoding chondroitinase ABC obtained from *Proteus vulgaris.*

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the present invention features a method for treating wounds comprising the step of administering an effective amount of a carbohydrate-active enzyme. Preferred carbohydrate-active enzymes of the present invention include but are not limited to chondroitinases and hyaluronidases. The carbohydrate-active enzymes may be administered by, for example, a pharmaceutical composition, transfer vectors or by transformed cell lines. The method is particularly applicable to mucosal wounds but also is effective for other types of wounds. The method is expecially applicable for burn wounds and wounds caused by trauma including surgical wounds.

In a second aspect the present invention features pharmaceutical compositions for treating wounds. The pharmaceutical compositions in accordance with the present invention comprise an effective amount of at least one carbohydrate-active enzyme. Preferred carbohydrate-active enzymes of the present compositions include but are not limited to chondroitinases and hyaluronidases. The compositions are particularly applicable to treating burn wounds but are also effective for other types of mucosal wounds such as those caused by trauma including surgical wounds. In some preferred embodiments, the composition comprising a carbohydrate-active enzyme is administered by topical, cutaneous or mucosal administration. In other preferred embodiments, the carbohydrate-active enzyme may be administered by cellular transformation vectors containing nucleic acid sequences encoding the carbohydrate-active enzymes.

An aspect of the present invention is to provide compositions for treating wounds such as burn wounds and trauma wounds, where the compositions are formulated so as to be adapted to the specific route of administration. In some embodiments, the present invention provides for administering the enzyme by topical cutaneous or mucosal administration. In other embodiments, the present invention provides for administering the enzyme by way of cellular transformation vectors comprising nucleic acid sequences encoding therapeutic carbohydrate-modifying enzymes.

In some preferred embodiments, the pharmaceutical compositions comprise a glycan-cleaving enzyme. Therapeutic enzymes may be administered in a number of ways such as intravenous, intralesional, topical, intranasal, inhalation or mucocutaneous administration. Another aspect of the subject invention is to provide for the administration of the enzyme by

formulating it with a pharmaceutically-acceptable carrier which may be a solid, a semi-solid or a liquid, and such compositions comprise a further aspect of the invention. Examples of pharmaceutical compositions include drops such as nasal drops, compositions for topical application such as ointments, jellies, creams and suspensions, aerosols for inhalation, nasal spray, liposomes, etc. Usually the enzyme will comprise between 0.05 and 99% or between 0.5 and 99% by weight of the composition, for example between 0.5 and 20% for compositions intended for injection and between 0.1 and 50% for compositions intended for topical administration.

When therapeutic enzymes are administered in the form of a subcutaneous implant, the compound is suspended or dissolved in a slowly dispersed material known to those skilled in the art, or administered in a device which slowly releases the active material through the use of a constant driving force such as an osmotic pump. In such cases administration over an extended period of time is possible.

To produce pharmaceutical compositions in this form of dosage units for topical application containing a therapeutic enzyme, the enzyme may be mixed with a solid, pulverulent carrier, for example lactose, saccharose, sorbitol, mannitol, a starch such as potato starch, corn starch, amylopectin, laminaria powder or citrus pulp powder, a cellulose derivative or gelatine and also may include lubricants such as magnesium or calcium stearate or a Carbowax or other polyethylene glycol waxes. Dyestuffs can be added to these formulations, for example, to distinguish between different contents of active substance.

Therapeutic carbohydrate-active enzymes of the subject invention may also be administered parenterally such as by subcutaneous or intralesional injection or by sustained release implant. In subcutaneous or intralesional injection the therapeutic enzyme (the carbohydrate-active enzyme) may be dissolved or dispersed in a liquid carrier vehicle. For intralesional administration the active material may be suitably admixed with an acceptable vehicle, preferably of the vegetable oil variety such as peanut oil, cottonseed oil and the like. Other parenteral vehicles such as organic compositions using solketal, glycerol, formal, and aqueous parenteral formulations may also be used.

For intralesional application by injection compositions may comprise an aqueous solution of a water soluble pharmaceutically acceptable salt of the active acids according to the invention, desirably in a concentration of 0.5-10%, and optionally also a stabilizing agent

and/or buffer substances in aqueous solution. Dosage units of the solution may advantageously be enclosed in ampoules.

The pharmaceutical compositions are preferably in the form of an ointment, gel, suspension, cream or the like. The amount of active substance may vary, for example between 0.05- 20% by weight of the active substance. Such pharmaceutical compositions for topical application may be prepared in known manners by mixing the active substance with known carrier materials such as isopropanol, glycerol, paraffin, stearyl alcohol, polyethylene glycol, etc. The pharmaceutically acceptable carrier may also include a known chemical absorption promoter. Examples of absorption promoters are, e.g., dimethylacetamide (U.S. Patent No. 3,472,931), trichloro ethanol or trifluoroethanol (U.S. Patent No. 3,891,757), certain alcohols and mixtures thereof (Great Britain Patent No. 1,001,949). A carrier material for topical application to unbroken skin is also described in the Great Britain patent specification No. 1,464,975, which discloses a carrier material consisting of a solvent comprising 40 70% (v/v) isopropanol and 0 60% (v/v) glycerol, the balance, if any, being an inert constituent of a diluent not exceeding 40% of the total volume of solvent.

The dosage at which the therapeutic carbohydrate-active enzyme containing pharmaceutical compositions are administered may vary within a wide range and will depend on various factors such as for example the severity of the infection, the age of the patient, etc., and may have to be individually adjusted. As a possible range for the amount of therapeutic enzyme which may be administered per day, about 0.1 mg to about 2000 mg or from about 1 mg to about 2000 mg is provided.

The pharmaceutical compositions containing the therapeutic carbohydrate-active enzymes may suitably be formulated so that they provide doses within these ranges either as single dosage units or as multiple dosage units. In addition to containing a therapeutic enzyme (or therapeutic enzymes), the subject formulations may contain one or more substrates or cofactors for the reaction catalyzed by the therapeutic enzyme in the compositions. Therapeutic enzyme containing compositions may also contain more than one therapeutic enzyme.

The therapeutic enzymes employed in the present methods and compositions may also be administered by means of transforming patient cells with polynucleic acids encoding the therapeutic carbohydrate-active enzyme when the therapeutic enzyme is a protein or

ribonucleic acid sequence. The therapeutic carbohydrate-active enzyme encoding sequence may be incorporated into a vector for transformation into cells of the subject to be treated. The vector may be designed so as to integrate into the chromosomes of the subject, e.g., retroviral vectors, or to replicate autonomously in the host cells. Vectors containing therapeutic enzyme encoding nucleotide sequences may be designed so as to provide for continuous or regulated expression of the therapeutic enzymes. Additionally, the genetic vector encoding the therapeutic enzymes may be designed so as to stably integrate into the cell genome or to only be present transiently. The general methodology of conventional genetic therapy may be applied to polynucleotide sequences encoding therapeutic enzymes. Reviews of conventional genetic therapy techniques can be found, in Friedman, *Science* 244:1275-1281 (1989); Ledley, *J Inherit Metab Dis* 13:587-616 (1990); Tososhev *et al.*, *Curr Opinions Biotech* 1:55-61 (1990).

In another aspect, the invention features cDNA sequences for carbohydrate-active enzymes, and cDNA expression systems for using various DNA sequences to produce mammalian carbohydrate-active enzymes for a variety of uses. The cDNA expression systems of the present invention provide a practical method for making carbohydrate-active enzymes having biological activity and in amounts which make the enzymes available for practical therapeutic use. Preferred carbohydrate-active enzymes of the present invention include but are not limited to chondroitinases and hyaluronidases.

The invention further includes recombinant plasmids comprising the cDNA of mammalian carbohydrate-active enzymes. Such plasmids are adapted for expression in eukaryotic cells and contain the regulatory elements necessary for expression of carbohydrate-active enzyme cDNA in eukaryotic cells.

The invention also includes transformed cells comprising a heterologous DNA sequence that encodes for a carbohydrate-active enzyme or a biologically active fragment or mutant thereof. Such a heterologous DNA sequence may be incorporated into a plasmid. In preferred embodiments, the transformed cells are eukaryotic cells and may be selected from the group consisting of mammalian cells, immortalized mammalian cells, fungi and yeasts.

In yet another aspect, the present invention features methods for producing a carbohydrate-active enzyme or a biologically active fragment or mutant thereof comprising the steps of culturing a transformed cell comprising a DNA sequence encoding for a

carbohydrate-active enzyme or a biologically active fragment or mutant thereof in a suitable nutrient medium and isolating the carbohydrate-active enzyme or a biologically active fragment or mutant thereof. In a preferred embodiment, the present invention features methods for obtaining a glycosaminoglycan-cleaving enzyme from *Proteus vulgaris*.

In another aspect, the present invention features a kit for treating wounds comprising a pharmaceutical composition having at least one carbohydrate-active enzyme therein.

The methods and compositions of the present invention invention differ substantially from other forms of medical therapy for wounds because conventional therapy methods feature proteolytic enzymes. This has a significant disadvantage because the mechanism of action of proteolytic enzymes provides significant toxicity and side effects in recipients.

The following definitions are provided to further clarify the present invention. The invention is not intended to be limited thereby. Those skilled in the art will understand that there are further meanings known to those skilled in the art for the terms used, and the examples are not intended to be exhaustive.

The term "carbohydrate-active enzyme" as used herein is intended to include any enzyme which alters a carbohydrate by reducing, oxidizing, cleaving or altering the chemical structure thereof. The term is specifically intended to encompass carbohydrate reducing enzymes. Examples of such enzymes include glycosaminoglycan reducing enzymes such as hyaluronidases, chondroitinases, dermatanases, heparanases, heparinases and keratanases. These specifically include endoglycosidases and exoglycosidases. Preferred carbohydrate-active enzymes include chondroitinases and hyaluronidases.

The term "mucocutaneous wound" as used herein is intended to include any wound which features alteration to the normal structure or cellular composition or cellular architecture of the integument including the epidermis, endodermis or underlying connective tissue.

The term "treating" as used herein is intended to include performing any steps or providing any agents which may alter the healing process of wounds by, for example, expediting exfoliation of damaged or dead tissues or cells, increasing vascular infiltration, and expediting the breakdown or removal of damaged cells or fragments thereof.

EXAMPLES OF THE PREFERRED EMBODIMENTS

The following examples are provided for illustration only. The invention is not intended to be limited thereby. Those of skill in the art may make many variations within the scope of the appended claims.

EXAMPLE 1

Glycosaminoglycans can be measured accurately in human skin.

Procedure: Measurement of glycosaminoglycans in skin is assessed using FACE technology (Glyko, Inc., Novato, CA). Skin samples are homogenized in a saline solution or subjected to protease digestion using collagenase (Sigma Chemical Co, St. Louis MO), and the supernatant is incubated with CPC reagent (cetylpyridinium chloride) to precipitate high molecular weight glycosaminoglycans (GAGs). The CPC precipitate is then washed and re-precipitated in LiCl and ethanol. GAGs are then digested with chondroitinase ABC (Seikagaku Chemical Company, Tokyo, Japan), labeled with fluorophore, and loaded onto FACE gels. The carbohydrates are separated by electrophoresis for approximately 1.5 hours followed by imaging of the gel. Unique chondroitin sulfate disaccharide bands are then quantified using the FACE imaging system provided by Glyko, Inc. Electronic images of fluorescent bands present on the gel are acquired by a CCD (charge-coupled-device) and processed into a digital image which is then displayed on a computer screen using FACE Imaging Software. The software allows for band quantification and band pattern recognition compared to a ladder of glucose polymers which are run in a single lane of the gel along with unknowns.

Results: Results of these studies are shown in Figures 1-6. Figure 1 represents the monosaccharide analysis of normal skin. Figure 2 represents the monosaccharide analysis of eschar from full-thickness burns. Figure 3 represents the glycosaminoglycan composition of normal skin. Figure 4 represents the glycosaminoglycan composition of the outer layer of normal skin. Figure 5 represents the glycosaminoglycan composition of the inner layer of normal skin. Figure 6 represents the glycosaminoglycan composition of total eschar.

EXAMPLE 2

Preparation of Chondroitinase ABC (Chondroitin ABC lyase; Chondroitin ABC eliminase; EC 4.2.2.4) from *Proteus vulgaris*

Procedure: Chondroitinase ABC is purified from culture supernatant of *Proteus vulgaris* grown on recommended media (Saito *et al.*, *J.Biol. Chem.* 243: 1543 (1968); Oike *et al.*, *J. Biol. Chem.* 257: 9751 (1982)). The supernatant from culture broth is precipitated with ammonium sulfate followed by DEAE-Cellulose chromatography. The material is prepared as a lyophilized powder with bovine serum albumin (less than 1 mg/50 U) added as a stabilizer.

The purified enzyme catalyzes the eliminative cleavage of N-acetylhexosaminide linkages in chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, chondroitin, and hyaluronic acid, yielding mainly disaccharides with Δ4-hexuronate at the non-reducing ends. The enzyme does not act on keratan sulfate, heparin, and heparan sulfate. The initial rates of enzymatic degradation of chondroitin sulfate C, dermatan sulfate, chondroitin and hyaluronic acid are 1.0, 0.4, 0.2, and 0.02, respectively, relative to the rate of chondroitin sulfate A degradation. The enzyme can be used for selective removal of the chondroitin sulfate or dermatan sulfate side chains from proteoglycans, yielding a protein-enriched core molecule.

Results: The results are summarized by the gel represented in Figure 7. Lane 1 represents a quantification control. Lane 2 has no enzyme control. Lane 3 demonstrates Chondroitinase ABC digests GAGs present in skin. Lane 4 represents the disaccharide standards against which chondroitinase ABC effectively digests.

SPECIFICATIONS:

Specific Activity	One unit is defined as the quantity of the enzyme that catalyzes the formation of 1 micromole of unsaturated disaccharide from chondroitin 6-sulfate per minute at 37 degrees C, pH 8.0 (Yamagata *et al.*, *J. Biol. Chem.* 243: 1523 (1968)).
Optimum pH	8.0 (for chondroitin sulfate) 6.2 (for hyaluronic acid)
Molecular weight	120,000- 145,000 (gel filtration)
Activators	Acetate (0.05M)
Inhibitors	Zn++(0.001M ZnCl inhibits by 100%)

Heparin (an equimolar amount of heparin in inhibits by ca. 70%)

EXAMPLE 3

Preparation of Chondroitinase B from *Flavobacterium heparinum*

The chondroitinase B sequence was retrieved ftom *Flavobacterium heparinum* (ATCC 13125) DNA by PCR amplification. Primers were designed based on a sequence previously deposited in Genbank (Accession U27584). The signal peptide was not included within the amplified coding sequence. Primer 5'-terminal restriction sites allowed direct cloning into the thioredoxin-fusion plasmid pThioHis (Invitrogen) cut with NcoI and EcoRI. Expression was optimized in *E. Coli* strain BL21.

Materials

The following materials were used:

For Cell Culture	LB/carbenicillin plate with pThioHisChondB in BL21 cells 50 ml sterile LB/carbenicillin (60 µg/ml) in a 250 ml flask 4 x 1.0 L sterile LB/carbenicillin in 2.8 L flasks with >4 cm stir bars 20 ml 200 mM IPTG 4 stir-plates, 4 large trays (for ice), 4 styrofoam platforms 2 x 500 ml centrifuge bottles
For Lysis	50 mM Tis-HCl, pH 8.0, 5 mM imidazole, 1 mM EDTA, 1 mM Pefabloc SC, 2 µg/ml Leupeptin Dry ice-ethanol bath 2 x 35 ml Oak Ridge centrifuge tubes
Column	Heparin-acrylic bead column (10-20 ml/L culture media) 500 ml 50 mM Tris-HCl, pH 8.0 40 ml 50 mM Tris-HCI, pH 8.0,150 mM NaCl

Procedure

The following procedures were followed:

Preparation

2.8 L flasks were autoclaved with LB media and stir bars. Carbenicillin was added after cooling. The media can be stored at room temperature for one to two days. A heparin

bead column was washed with 2 column volumes of >1 M NaCl and at least 3 column volumes of 50 mM Tris-HCl, pH 8.0. A single-colony of pThioHisChondB in BL21 cells were inoculated into 100 ml of LB/carbenicillin. The cells were incubated at 37°C overnight with 250 rpm shaking. Subculture if stationary.

Cell Culture

20 ml of overnight culture were seeded into each 2.8 L flask. The flasks were shaken at 37°C with 250 rpm shaking until 0.5<OD550<0.7. The bottles were immediately transferred to ice-filled trays on stir-plates and stirred at moderate speed until the culture temperature was <10°C (about an hour). A few drops of Antifoam 289 were added at this stage to prevent frothing of cultures. 5 ml of 200 mM IPTG were added to each culture, and the ice trays were removed after about 5 minutes. The cultures were stirred at room temperature for three hours. Styrofoam was placed between bottles and stir-plates. The cultures were pelleted in 2 x 500 ml bottles (8000 rpm for 10 minutes). The two pellets were weighed and (combined weight between 5-7 grams).

Cell Lysis

The pellets were resuspended in a total of 80 ml lysis buffer (protease inhibitors added fresh) and transferred to 2 x 50 ml polypropylene Falcon tubes. The tubes were then vortexed to insure that cells were completely resuspended and that no clumps remained. The cells were then immediately frozen by immersion in a dry ice-ethanol bath. At least 10 minutes were allowed for complete freezing to occur. The cell solution was then quickly thawed by immersing the tubes in a 37°C bath and shaking. The thawed cells were placed on ice and sonicated with a Branson 450 microtip using an output of 3-4 and a duty cycle of 100% for at least 3 x 15 seconds. The freezing, thawing, sonicating steps were repeated four times. The lysate was transferred to 2 x 35 ml Oak Ridge tubes and clarified by spinning at > 10,000 rpm for at least 30 minutes. The supernatant was transferred back to a single 50 ml Falcon tube and supplemented with MgCl2 to 10 mM, RNase A to 10 μg/ml, and DNase I to 10 μg/ml. The tube was rocked at 4°C for 30 minutes. The weight of the pellet after lysis was determined to be about 1/3 that of the original cell pellet. 5 μL and 10 μL supernatant were assayed for activity against dermatan sulfate.

Column Chromatography

The entire nucleic acid-depleted supernatant was added onto a heparin column, and the sample was allowed to flow through column at a rate of <2.0 ml/minute. 5 µL of the flow-through was assayed to ensure capture of the chondroitinase. The column was washed with 50-100 ml 50 mM Tris-HCI, pH 8.0. The wash was checked for activity to confirm that the majority of the chondroitinase remained bound. The chondroitinase was eluted with 40 ml or less of 50 mM Tris-HCI, pH 8.0, 150 mM NaCl. The activity of the eluate was determined. The eluate was supplemented with a stabilizing agent. Successful additives to the column media include BSA or ovalbumin at 0.05%. A 10% protein gel of the pre-column supernatant, flow-through, wash, and eluate fractions was run for SDS-PAGE. The molecular weight of thiochondroitinase B is about 66 kD. The column can be regenerated by washing with >1 M NaCl and reequilibrating with the 50 mM Tris-HCI, pH 8.0. The matrix can be stored in Tris buffer containing 0.02% thimerosal.

Chondroitinase B Quality Control Protocol

The activity of chondroitinase B in cleaving N-acetylgalactosamine linked to L-iduroni acid in dermatan sulfate may be determined by the following protocol. Chondroitin Sulfate B was obtained from Sigma and used as the substrate.

Materials Required:
Spectrophotometer
Chondroitin Sulfate B at 20 mg/ml in deionized water
50 mM Tris-HCI, pH 8.0 (Trizma Base, Sigma Cat# T-8404) Quartz cuvettes
P-1000 pipetman
P-20 pipetman

Chondroitin Sulfate B is dissolved in deionized water to a concentration of 20 mg/ml. A quartz cuvette was filled to a final volume of 1.0 ml comprised of 50 mM TrisHCI, pH 8.0 and Chondroitin Sulfate B, at a final concentration of 100 µg/ml. After warming up the spectrophotometer, the wavelength on the spectrophotometer was set to 232 nm. Contents were added to a quartz cuvette and mixed by inverting the cuvette. The cuvette was placed inside. After 1 minute the absorbance reading was recorded. The reactions were stopped by adding 480 µl of Stop Reagent. Incubation for 10 minutes at 37°C and then centrifugation for 5 minutes. 400 µl of supernatant was pipetted into a new tube and 600 µl of Assay Buffer

was added. These were mixed and absorbance immediately read (A574) of the sample against blank at room temperature. Absorbance readings should be less than 0.02 to pass the protease test.

Calculating the Activity

The enzyme activity was calculated using the following formula:

$$\frac{OD232 \times 106}{time(min) \times enzyme\ volume(\mu l) \times 5100} \quad \mu mole/min/\mu l$$

Quality Control Assay for Detecting Contaminating Enzyme Activity

Contaminating Chondroitinase AC activity may be evaluated with the assay above, using the following substrates in place of dermatan sulfate:

Chondroitin Sulfate A
Chondroitin Sulfate C
Hyaluronic Acid

Any absorbance reading less than 0.002 can be considered background and is acceptable.

Quality Control Assay for Detecting Protease Activity

Protease detection is based on the release of resorufin by proteolysis of tagged peptides, as detailed below (Boehringer Mannheim):

The following solutions were prepared:

A. Substrate Solution: 0.4% Casein, resorufin-labeled (w/v) in redistilled water
B. Incubation Buffer: 0.2 M Tris-HCl, pH 7.8, 0.02 M $CaCl_2$,
C. Sample Solution: Chondroitinase B enzyme at the appropriate final concentration
D. Stop Reagent: 5% Trichloroacetic acid (w/v) in redistilled water
E. Assay Buffer: 0.5 M Tris-HCl, pH 8.8.

Set up these reactions in 1 ml reaction vessels and incubate overnight at 37°C.

	Sample Blank	Sample
Substrate Solution	50 µl	50 µl
Redistilled Water	100 µl	90 µl
Sample Solution	–	10 µl

EXAMPLE 4

Useful Formulations of Chondroitinase ABC

We prepared a glycerin-based hydrogel. The hydrogel formulation was evaluated for stability, release of enzyme by diffusion cell and agar plate and for pilot sterility feasibility. Variations of the basic hydrogel were evaluated for stability and release. The variations evaluated include buffer concentration, hydroxyethyl cellulose concentration, glycerin concentration, presence of various additives and the use of carbopol as a gelling agent.

We have determined that chondroitinase ABC is relatively unstable in glycerin alone without the addition of phosphate buffer. Preliminary data suggest that chondroitinase ABC stability is enhanced with the higher concentration of phosphate buffer.

We have determined that a glycerin-based hydrogel is stable at 5°, room temperature and 40°C for 3 months. The same formulation is also stable at 5 and 30°C for one month. The addition of urea or carbopol (pH adjusted with 50% trolamine instead of phosphate buffer) to the hydrogel formulation causes significant instability of chondroitinase ABC at 30 and 40°C. (65 and 45% loss respectively from T=0 after 1 month at 40°C). The addition of benzyl alcohol or dioctyl sodium sulfosuccinate appears to cause instability of chondroitinase ABC but to a lesser degree. (33% loss from T=0 after 1 month at 40°C). A higher concentration of phosphate buffer (300 mM instead of 50 mM) appears to enhance the stability of chondroitinase ABC in the hydrogel formulaion.

A hydrogel formulation of chondroitinase ABC was prepared. It has a sustained release for at least 24 hours with 25 to 35% of the enzyme activity released. Further sustained release studies on agar plate with formulation with two concentrations of chondroitinase ABC (100 & 500 U/g) vs. a solution of chondroitinase ABC at 500 U/g. The study revealed increased zone of clearing (or area of hydrolysis) is seen with increased concentrations of chondroitinase ABC in hydrogel formulations after 24 hours.

A glycerin-based chondroitinase ABC hydrogel at 500 U/g was packaged into 3 gram Laminate tubes and irradiated with E-beam radiation at 10, 15, 20 and 30 kGy. A 25% loss of

activity occurred at 10 kGy and increased with higher doses of radiation to a 40% loss at 30 kGy.

A glycerin-based hydrogel having 300 mM phosphate buffer or alternatively having 50 mM phosphate buffer has proven to be an effective delivery formulation. Low levels of parabens or benzyl alcohol may be introduced as acceptable preservatives.

EXAMPLE 5

Carbohydrate-degrading enzymes degrade glycosaminoglycans in skin

Procedure: Skin samples from patients with burns and normal persons were obtained. For these analyses skin samples were stored at -70°C until the time of assay. Thawed samples were homogenized in a saline solution, and the GAGs were precipitated from 500 µl of supernatant by adding 0.5 ml of CPC (cetylpyridinium chloride) reagent (0.2M NaCitrate, 0.1% CPC, pH 4.8). The mixture was incubated for 30 minutes at 37°C. The CPC precipitate was recovered by centrifugation for 5 minutes at 14,000xg, and the supernatant was discarded. The CPC pellet was washed by dissolving the pellet in 67 µl 2M LiCl, adding 267 µl of cold ethanol and re-precipitating the CPC for 2 hours at 4°C. The washed CPC pellet was recovered by centrifugation for 5 minutes at 14,000xg and resuspended in 50 µl of H_2O. The residue was digested into disaccharides by adding 20 µl of chondroitinase ABC solution and incubating for 30 minutes at 37°C. The digest was dried in a centrifugal vacuum evaporator, and the disaccharides were labeled with a fluorescent tag by resuspending the residue in 5 µl of the fluorophore AMAC (2-aminoacridone), 0.15M in 15% acetic acid and 5 µl of 1M $NaCNBH_4$ in DMSO. The labeling reaction was complete in 16 hours at 37°C. The vial of labeled oligosaccharides was brought to 20µl in 10% glycerol, and 4 µl was placed in the lane of a FACE pre-cast polyacrylamide gel and electrophoresed for 1.5 hours followed by imaging of the gel. Unique chondroitin sulfate disaccharide bands were then quantified using the FACE imaging system provided by Glyko, Inc. Electronic images of fluorescent bands present on the gel were acquired by a CCD (charge-coupled-device) and processed into a digital image which was then displayed on a computer screen using FACE Imaging Software. The software allows for band quantification and band pattern recognition

compared to a ladder of glucose polymers which are run in a single lane of the gel along with unknowns.

Results: The results are provided in Figure 7. This figure demonstrates that the purified enzyme Chondroitinase ABC catalyzes the eliminative cleavage of N-acetylhexosaminide linkages in chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, chondroitin, and hyaluronic acid, yielding mainly disaccharides with Δ4-hexuronate at the non-reducing ends. The enzyme does not substantially act on keratan sulfate, heparin, and heparan sulfate. Lane 1 represents a quantification control. Lane 2 has no enzyme control. Lane 3 demonstrates that Chondroitinase ABC digests GAGs present in skin. Lane 4 represents the disaccharide standards that chondroitinase ABC effectively digests.

EXAMPLE 6

Enzymatic debridement of full thickness thermal burns in mice using chondroitinases and hyaluronidases.

Methods: Inbred BALB-c mice, 8-10 weeks of age were used in this study. After hair removal, the mice were anesthetized and narcotics given appropriately prior to a full thickness contact burn wound (approximately 30% total body surface area). A 2 X 2 cm hot template was applied to the dorsum of the mice with a pressure of 50 grams and immediately fluid resuscitated. Enzymatic treatment was done once a day until the wound was 95% clean and then closed with isograft. A surgical control group underwent a full thickness burn, immediate surgical excision and grafting. Santyl® (collagenase) treated full thickness burns were used as an enzyme control. The experimental burn wounds underwent topical treatment with a chondroitinase ABC (from *Proteus vulgaris*, Seikagaku, Tokyo Japan) prepared in sodium phosphate buffer pH 7.4. Wounds were cleaned with sterile gauze and normal saline to remove the eschar. Debridement was standardized to 20 strokes per wound each day. Wounds were considered clean after at least 95% of the eschar was removed. The end points of the study were days to wound cleaning (95% clean wound) and days to complete epithelialization (95% epithelialized).

Results: Enzymatic debridement with collagenase resulted in the partial dissolution of eschar until the wound was cleaned. Wound bleeding was noticed on the last day of treatment in most wounds. The collagenase group had a median of 4 days to clean the wound compared

to 5 days for chondroitinase ABC (p value not significant). Removal of eschar in the chondroitinase ABC group occurred as a single layer with clean separation of eschar at the interface of the burned and underlying unburned tissue. Greater than 50% of eschar was removed during the first three days of wound treatment in the chondroitinase ABC group. In the surgical control group, wounds were completely epithelialized with a median of 12 days after grafting. Wound closure of wounds treated with collagenase and chondroitinase ABC occurred in 25 and 14 days respectively. These results are demonstrated in Figure 8. The wounds treated with chondroitinase were cleaner than those treated with collagenase, had less bleeding and epithelialized sooner.

EXAMPLE 7

Enzymatic debridement of full thickness burns in pigs using chondroitinases and hyaluronidases.

A porcine burn model is used to obtain these data. The porcine burn model protocol is as follows:

Day 1: Male Yorkshire pig (50-70lbs) is sedated with Ketamine Hydrochloride (20 mg/kg and Acepromazine Maleate (0.5 mg/kg) by intramuscular injection. The animal is placed under isoflurane inhalation anesthesia to induce sleep but maintains spontaneous respiration while in the operating room. The animal is positioned on his belly. The back is then shaved with shears and further shaved with a razor. The skin surface is prepped with a soap scrub and dried.

Four groups of three burn wounds are mapped out over both of the animal's flanks using an ink pen. After the animal is repositioned to provide a flat flank surface, full thickness thermal wounds are then created at these designated spaces. The burning is performed using metal templates 3.5cm in diameter that are heated in boiling water (100 ° C). These heated discs are then placed on the animal's skin with minimal but equal pressure for 35 seconds.

The wounds are then covered in OpSite shectina (Smith-Nephew). An elastic nettina sleeve (Durr Medical; Montgomerv, AL) is then placed over the animal's torso, and bandaging tape is applied at both ends to hold the dressing in place after the pig resumes

normal daily activities. The animal is taken off isoflutane inhalation and returned to its cage where it is closely monitored until full recovery has taken place.

Day2: The pig is again sedated and anesthetized as outlined above. The OpSite covering is removed and the animal's flank is wiped down with saline. Attention is turned to the previously made burn wounds grouped in sets of three and randomized to receive a blinded enzyme formula. In each burn group, one wound receives the specific enzyme formula directly, another is treated with this formula after the eschar has been aerated, and the final wound is treated with the formula during the aerating process. A Permark tattooing device (Micropigmentation Devices Inc.; Edison, NJ) with a 14 needle tip is used to perforate or "aerate" the eschar surface of the above selected wounds. After these treatments, OpSite sheeting is reapplied separately to the individual sets of wounds. The pig is then dressed in the usual fashion and returned to its cage to recover.

Day3. The pig is sedated and anesthetized in the usual fashion. The bandages are removed, and the wound drainage is cleaned from the protected surfaces with saline. Light debridement is then performed to each wound using dry clean gauze. Photographs are then taken of each wound group to document the effects of each enzyme formula. The wounds are then excised from each flank, sectioned, and fixed in 4% paraformaldehyde. The animal is humanely euthanized with an intracardiac injection of Pentobarbital sodium (6grains/ml/10lbs).

The above tissues are trichrome stained for histologic evaluation and assessment of the efficacy of enzymatic debridement of the full thickness burn.

Results

On postburn day 3, wounds are divided into three groups: a) Phosphate-buffered Saline pH 7.0, b) hyaluronidase, and c) chondroitinase ABC. On postburn day 4 (after 24 hours of hydrolysis or a single treatment), one wound from each treatment group is removed by a dermatome on a full-thickness setting. On postburn day 5 (after 48 hours of hydrolysis or two successive topical treatments), one wound from each treatment group is removed for analysis. This removal procedure is repeated on postburn days 6 and 7 after burn injury, which represents 72 and 96 hours of hydrolysis or three and four topical treatments, respectively. Additional wounds from each treatment group continue to receive placebo formulation after enzyme treatments are concluded and are removed after 10 days of healing.

Wound tissues including normal margins from the edges and underlying normal dermis from these partial-thickness wounds are fixed in 10% neutral buffered formalin for 48 hours and embedded in paraffin wax. Representative sections (6 pm) are stained with Gomori's Trichrome and photographed with an Olympus Vanox AH light microscope (Olympus America, Inc., Lake Success, N.Y.).

Formulation A

Wounds receive 48 hours of hyaluronidase hydrolysis (two successive treatments) as described herein and are harvested and stained with Trichrome. A. photo is made at the wound edge so the extent of tissue removal may be appreciated. The dermal eschar shows extensive disruption. The hardened eschar is softened with topical treatment

Formulation B

Wounds receive 48 hours of phosphate buffered saline (two successive treatments) as described herein and are harvested and stained with Trichrome. A photo is made at the wound edge so the extent of tissue removal may be appreciated. The dermal eschar shows no evidence of disruption. The hardened eschar has not been softened with topical treatment. The epidermis at the surface is sloughing, and deeper hair follicles show extensive damage.

Formulation C

Wounds receive 48 hours of chondroitinase ABC hydrolysis (two successive treatments) as described herein and are harvested and stained with Trichrome. A photo is made at the wound edge so the extent of tissue removal may be appreciated. The dermal eschar shows extensive disruption. The hardened eschar is softened with topical treatment

At the dose tested (1400 units/gm vehicle), optimal hydrolysis of eschar from the surface of partial-thickness burns is evident after two successive treatments with hyaluronidase and chondroitinase ABC (Figures 9 and 11). By comparison, incomplete hydrolysis and nominal effects on eschar is observed after two treatments (48 hours of hydrolysis) within wounds receiving phosphate buffered saline (Figure 10). Wounds in the phosphate buffered saline group may be characterized as heat-fixed or desiccated *in situ*. At the surface of nontreated wounds, the epidermis remains in place, although upon closer examination, epithelial cells at the surface and those comprising deeper hair follicles show signs of necrosis.

Qualitative evaluation of granulation tissue (neodermis)

Histologic differences among the enzyme and phosphate buffered saline groups is most pronounced at postburn day 10. Enzyme treated wounds show robust ingrowth of granulation tissue into the tissue defect. The wound bed is entirely filled with an influx of fibroblasts, macrophages, and new capillaries. Staining of the newly synthesized matrix appears pale in contrast to the surrounding nonwounded dermis with its mature collagen fibrils. In enzyme-treated wounds, small pockets of granulation tissue are interspersed among mature collagen fibrils

Dose response evaluation

Both neodermal (granulation tissue) responses and re-epithelialization are evaluated as a function of dose response. By gross observation, all three of the vibriolysin doses tested remove nonviable tissue from the wounds. Quantitative histologic evaluations after 10 days of healing indicates a dose-responsive effect in the depth of the granulation tissue as a function of the enzyme concentration. The lowest dose tested shows a significantly greater granulation tissue formation compared to the PBS formulation. In addition, regrowth of the epidermis is unremarkable, and wounds are 100% resurfaced by post-burn day 10.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

WHAT IS CLAIMED:

1. A method for treating wounds comprising the step of administering an effective amount of a carbohydrate-active enzyme.

2. A method according to claim 1 wherein the carbohydrate-active enzyme is selected from the group consisting of a chondroitinase and a hyaluronidase.

3. A method according to claim 1 wherein the carbohydrate-active enzyme is administered by a pharmaceutical composition.

4. A method according to claim 1 wherein the carbohydrate-active enzyme is administered by a transfer vector.

5. A method according to claim 1 wherein the carbohydrate-active enzyme is administered by a transformed cell line.

6. A method according to claim 1 wherein the wound is a mucocutaneous wound.

7. A method according to claim 1 wherein the wound is a burn wound.

8. A pharmaceutical composition for treating a wound comprising an effective amount of at least one carbohydrate-active enzyme.

9. The pharmaceutical composition of claim 8 wherein the carbohydrate-active enzyme is selected from the group consisting of a chondroitinase and a hyaluronidase.

10. The pharmaceutical composition of claim 8 designed to be administered topically.

11. A pharmaceutical composition for treating a wound comprising a cellular transformation vector having a nucleic acid sequence encoding a carbohydrate-active enzyme.

12. A pharmaceutical composition according to claim 8 wherein the carbohydrate-active enzyme is a glycan-cleaving enzyme.

13. A method for treating wounds comprising the step of administering an effective amount of a polynucleic acids encoding a carbohydrate-active enzyme.

14. A cDNA sequence encoding a carbohydrate-active enzyme.

15. A cDNA sequence according to claim 14 encoding a carbohydrate-active enzyme selected from the group consisting of a chondroitinase and a hyaluronidase.

16. A recombinant plasmid comprising a cDNA sequence encoding a carbohydrate-active enzyme.

17. A transformed cell comprising a heterologous DNA sequence that encodes for a carbohydrate-active enzyme.

18. A transformed cell according to claim 17 wherein the transformed cell is selected from the group consisting of a mammalian cell, an immortalized mammalian cell, a fungus and a yeast.

19. A method for producing a carbohydrate-active enzyme comprising the steps of:

(a) culturing a transformed cell comprising a DNA sequence encoding for a carbohydrate-active enzyme in a suitable nutrient medium; and

(b) isolating the carbohydrate-active enzyme.

20. A method for obtaining a glycosaminoglycan-cleaving enzyme from *Proteus vulgaris* comprising the steps of:

(a) Culturing Proteus vulgaris in a suitable nutrient medium; and

(b) Isolating the glycosaminoglycan-cleaving enzyme.

21. A kit useful for treating wounds comprising a pharmaceutical composition having at least one carbohydrate-active enzyme therein.

22. A purified product from cultures of a *Proteus species* having the ability to degrade a glycosaminoglycans.

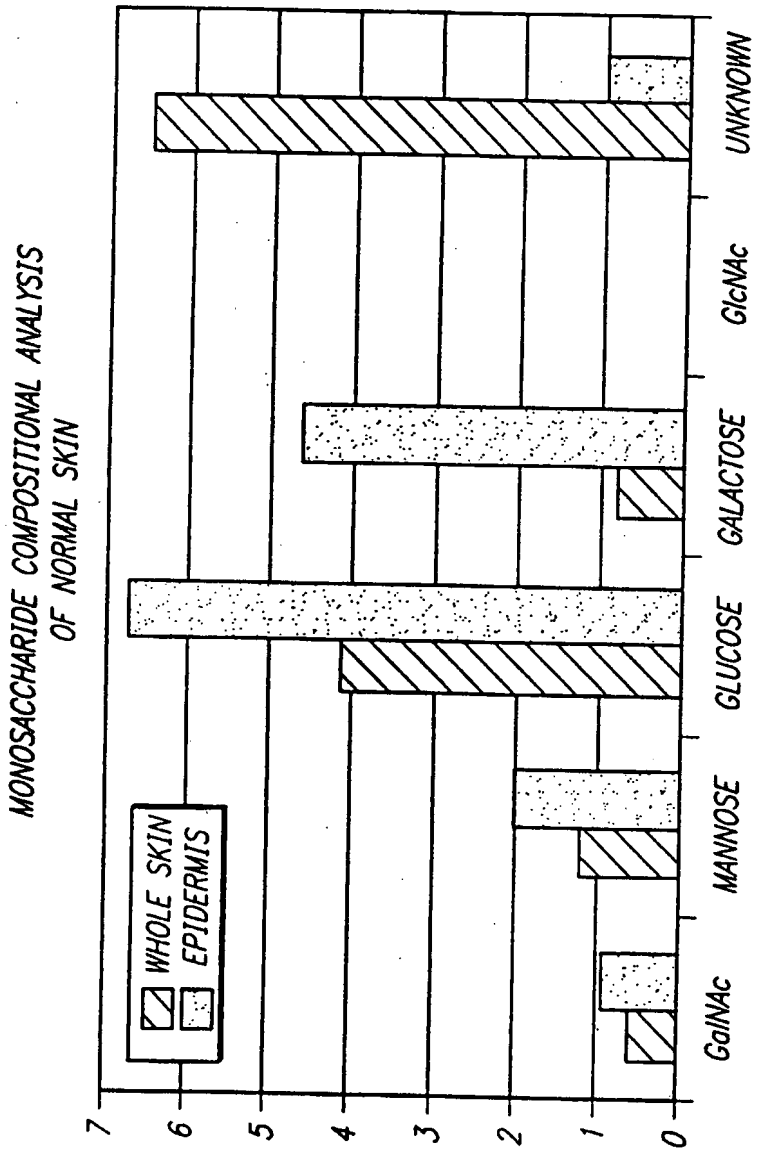

MONOSACCHARIDE COMPOSITIONAL ANALYSIS
OF NORMAL SKIN
WHOLE SKIN
EPIDERMIS
0
1
2
3
4
5
6
7
GalNAc
MANNOSE
GLUCOSE
GALACTOSE
GlcNAc
UNKNOWN

FIG. 1

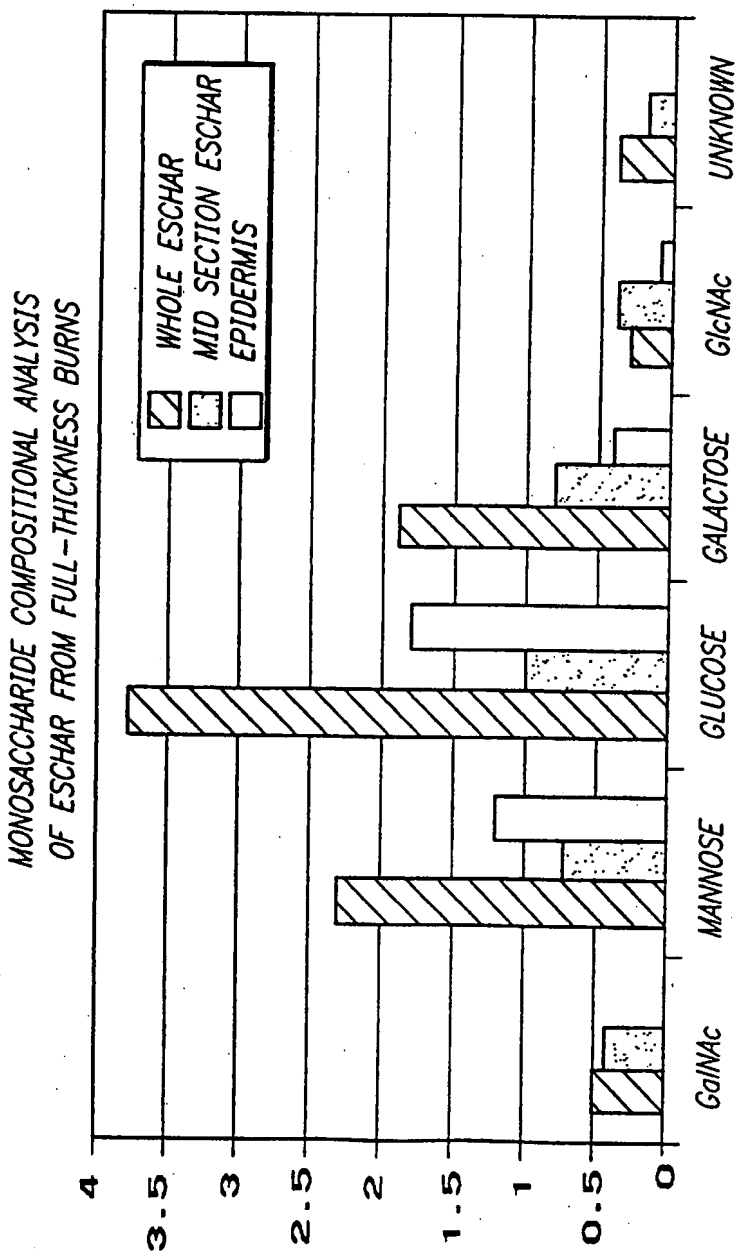

MONOSACCHARIDE COMPOSITIONAL ANALYSIS
OF ESCHAR FROM FULL-THICKNESS BURNS
WHOLE ESCHAR
MID SECTION ESCHAR
EPIDERMIS
4
3.5
3
2.5
2
1.5
1
0.5
0
GalNAc
MANNOSE
GLUCOSE
GALACTOSE
GlcNAc
UNKNOWN

FIG. 2

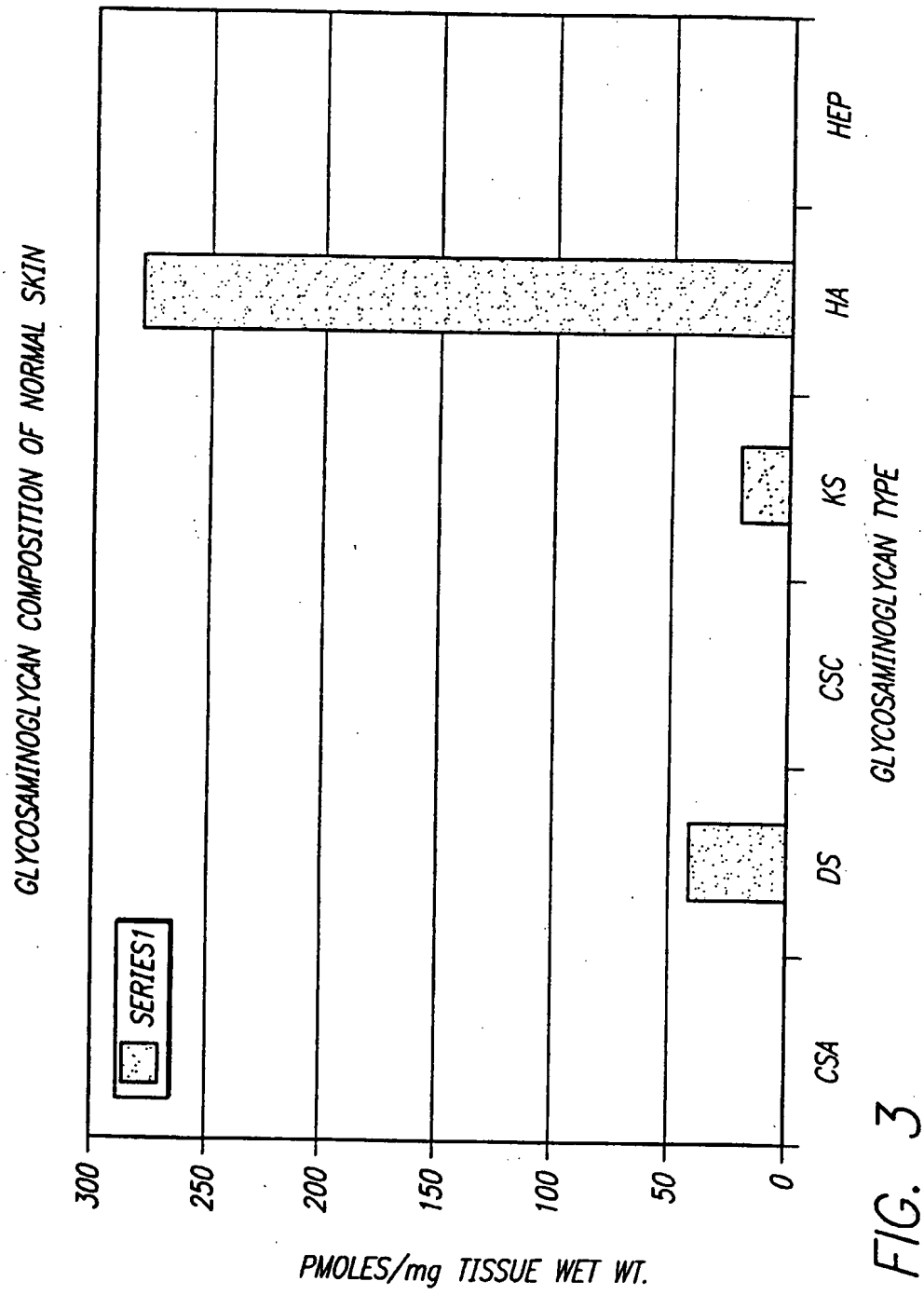

GLYCOSAMINOGLYCAN COMPOSITION OF NORMAL SKIN
SERIES1
300
250
200
150
100
50
0
PMOLES/mg TISSUE WET WT.
CSA
DS
CSC
KS
HA
HEP
GLYCOSAMINOGLYCAN TYPE

FIG. 3

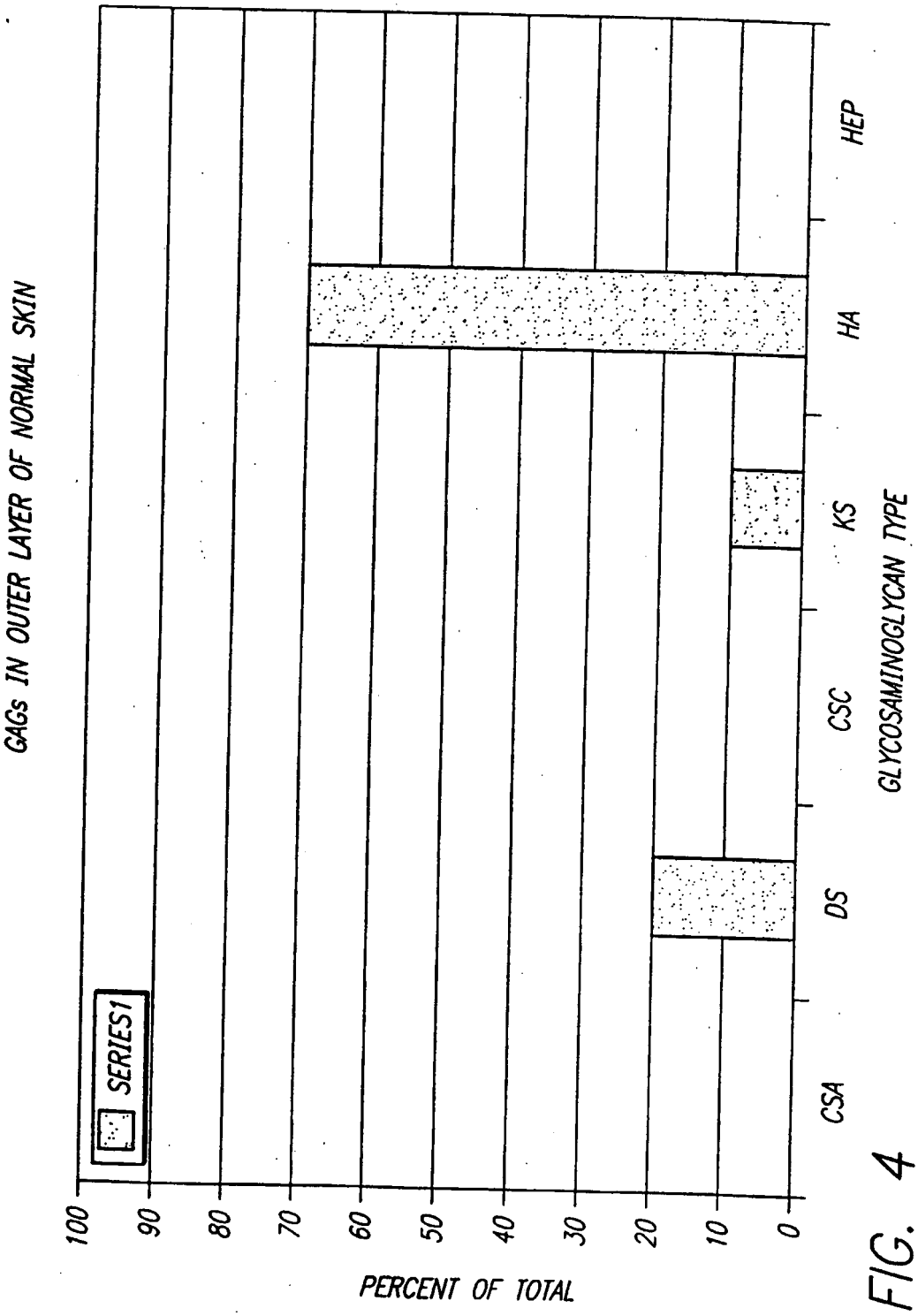

GAGs IN OUTER LAYER OF NORMAL SKIN
SERIES1
100
90
80
70
60
50
40
30
20
10
0
PERCENT OF TOTAL
CSA
DS
CSC
KS
HA
HEP
GLYCOSAMINOGLYCAN TYPE

FIG. 4

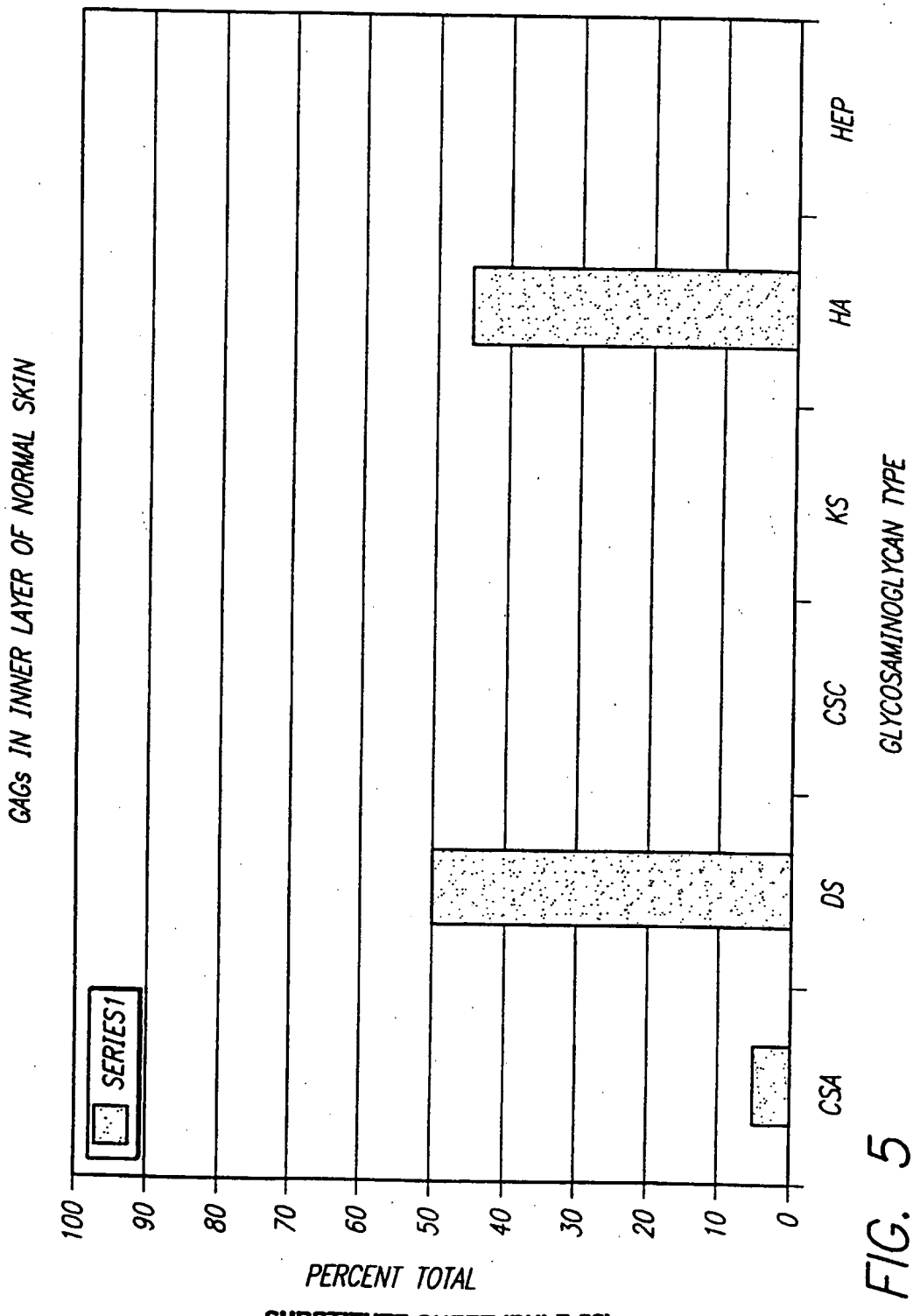

GAGs IN INNER LAYER OF NORMAL SKIN
SERIES1
100
90
80
70
60
50
40
30
20
10
0
PERCENT TOTAL
CSA
DS
CSC
KS
HA
HEP
GLYCOSAMINOGLYCAN TYPE

FIG. 5

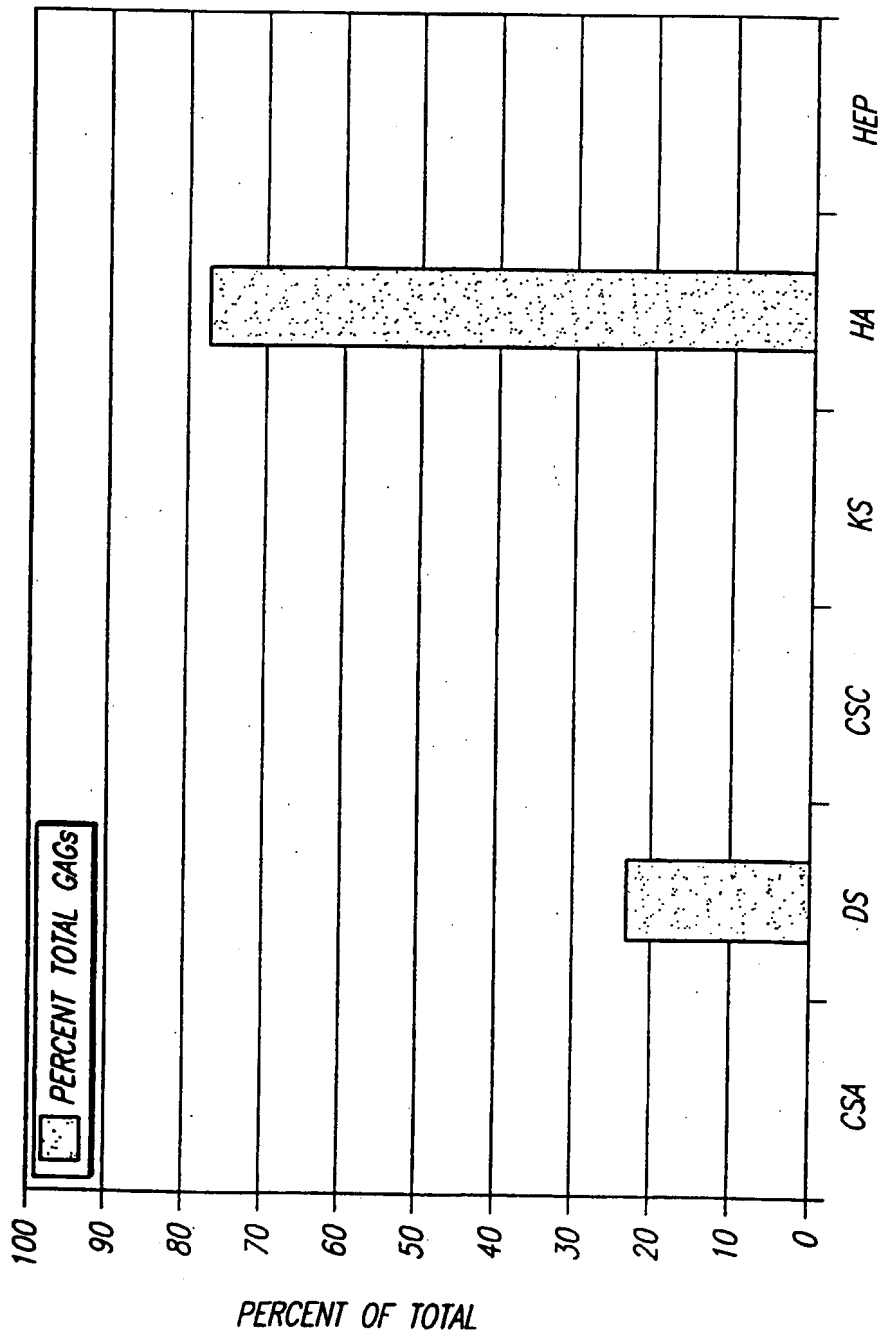
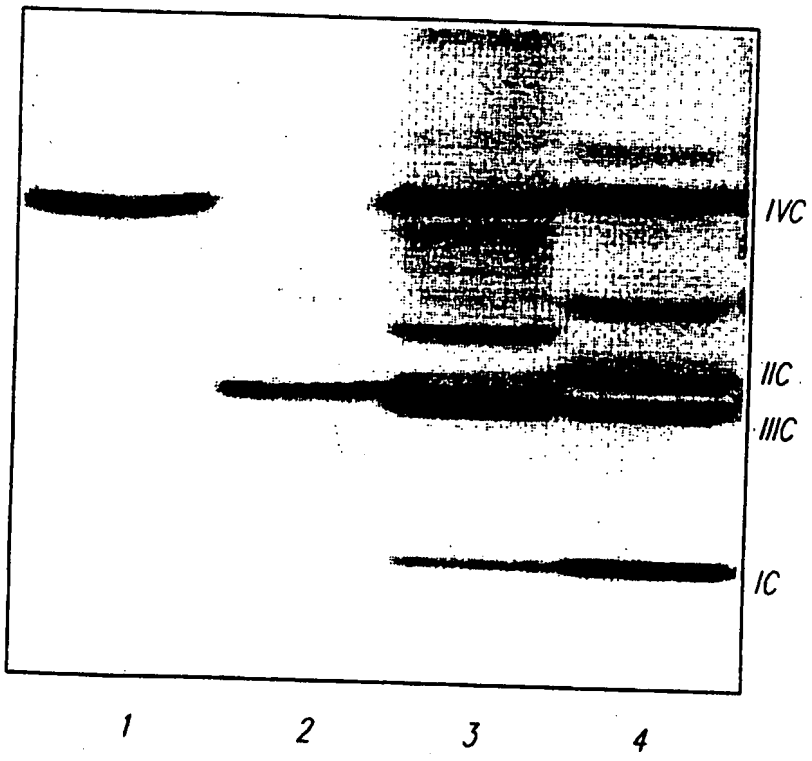

PERCENT TOTAL GAGs
100
90
80
70
60
50
40
30
20
10
0
PERCENT OF TOTAL
CSA
DS
CSC
KS
HA
HEP
GLYCOSAMINOGLYCAN TYPE

FIG. 6

DEGRADATION OF HUMAN SKIN GLYCOSAMINOGLYCANS BY CHONDROITINASE ABC



LANE 1 QUANTIFICATION CONTROL
LANE 2 MINUS ENZYME CONTROL
LANE 3 CHONDROITINASE ABC DIGESTION OF SKIN GAGs
LANE 4 CHONDROITIN SULFATE DISACCHARIDE STANDARDS

FIG. 7

FIG. 8A

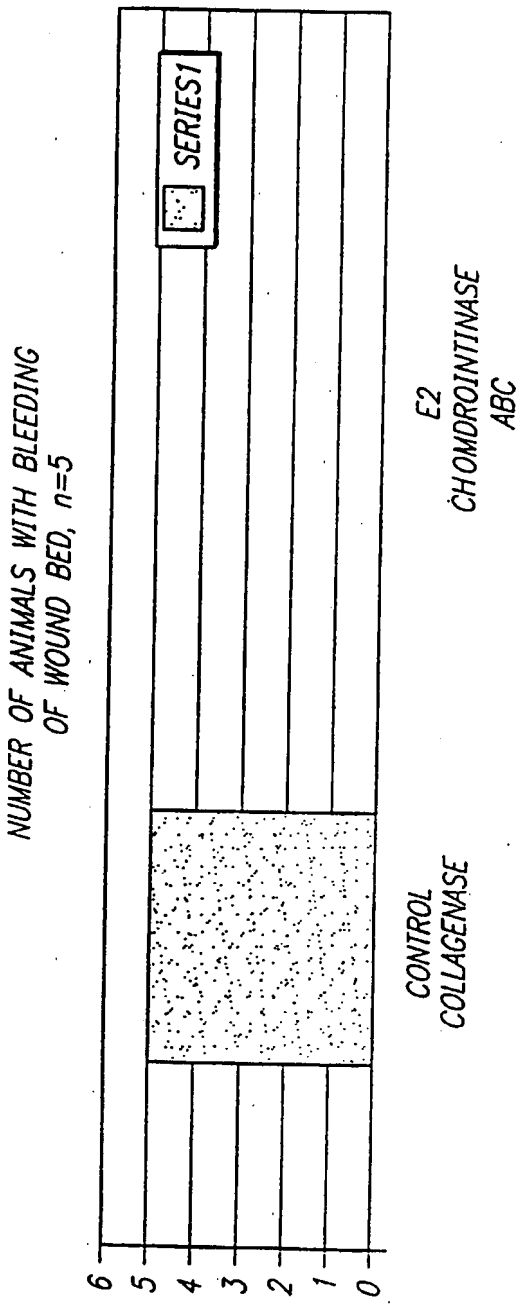
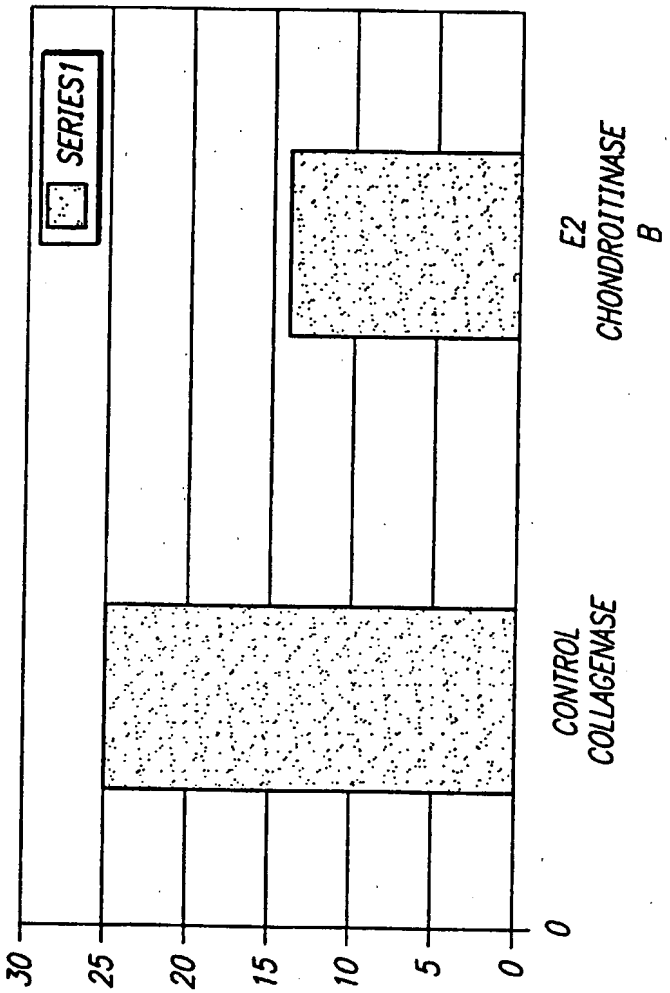

NUMBER OF ANIMALS WITH BLEEDING OF WOUND BED, n=5
SERIES1
CONTROL
COLLAGENASE
E2
CHOMDROINTINASE
ABC
6
5
4
3
2
1
0

FIG. 8B


DAYS TO COMPLETE EPITHELIALIZATION AND WOUND CLOSURE
SERIES1
E2 CHONDROITINASE B
CONTROL COLLAGENASE
30
25
20
15
10
5
0
0

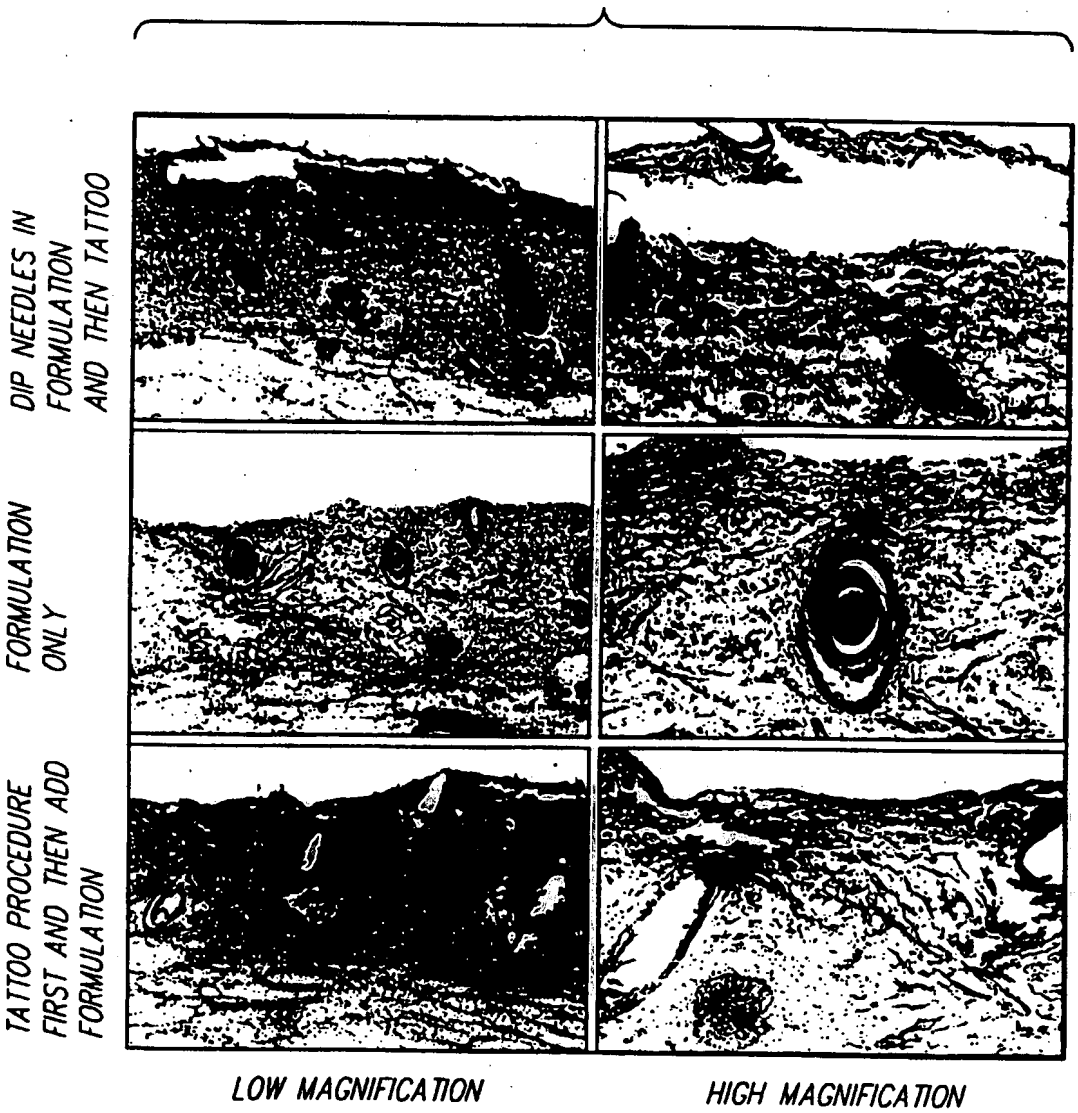

DIP NEEDLES IN FORMULATION AND THEN TATTOO
FORMULATION ONLY
TATTOO PROCEDURE FIRST AND THEN ADD FORMULATION
LOW MAGNIFICATION
HIGH MAGNIFICATION

FIG. 9

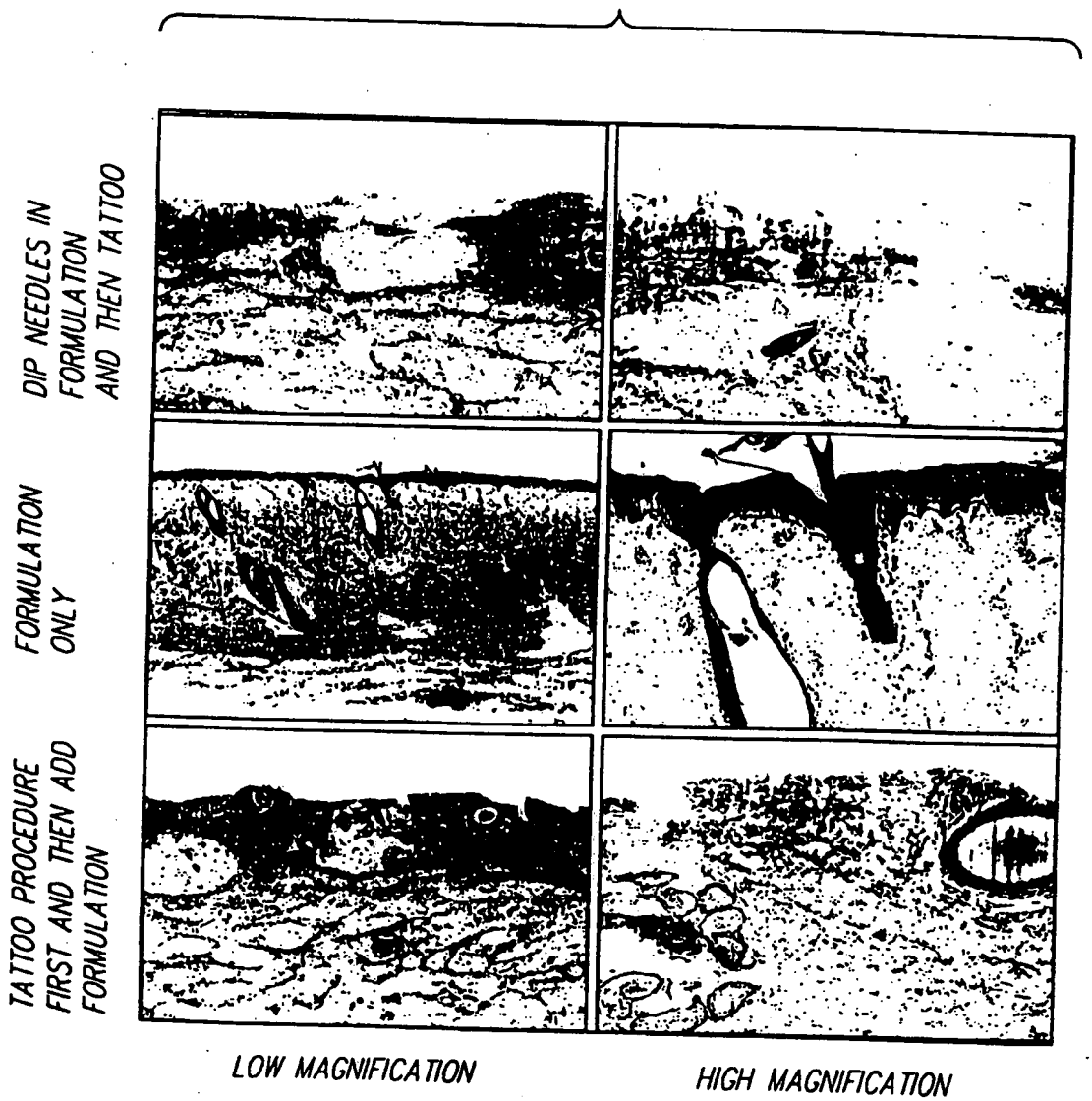

DIP NEEDLES IN FORMULATION AND THEN TATTOO
FORMULATION ONLY
TATTOO PROCEDURE FIRST AND THEN ADD FORMULATION
LOW MAGNIFICATION
HIGH MAGNIFICATION

FIG. 10

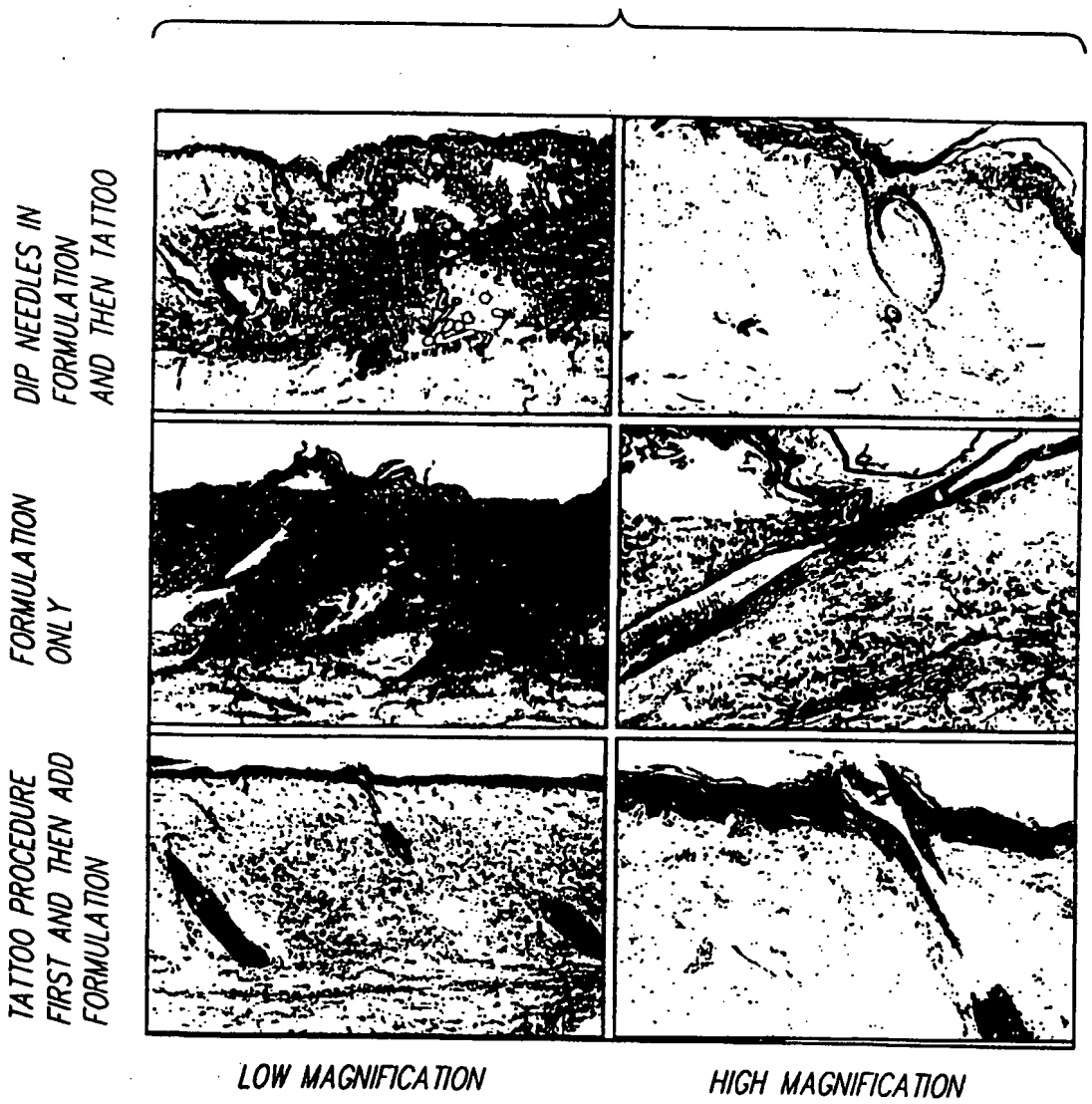

DIP NEEDLES IN FORMULATION AND THEN TATTOO
FORMULATION ONLY
TATTOO PROCEDURE FIRST AND THEN ADD FORMULATION
LOW MAGNIFICATION
HIGH MAGNIFICATION

FIG. 11

FIG. 12

CTTAAGGCAGATGGTGCAATCCTGGTTGATTTCTGGGCACACTGGTCGGTCCGTGCAAAATGATCG
CTCCGATTCTGGATGAAATCGCTTGACGAATATCAGGGCAAACTTGACCGTTGCAAAACTTGAACA
TCGATCACAACCCGGGCACTGCGCCGAAATATGGCATCCGTGGTATCCCGACTCTGCTGCTGTTCA
AAAACGGTGAAGTGGCGGCAACCAAAGTGGGTGCACTGTCTAAAGGTCAGTTGAAAGAGTTCCTCG
ACGCTAACCTGGCCGGCTCTGGATCCGGTGATGACGATGACAAGGTACCCATGGGACAGGTTGTTG
CTTCAAATGAAACTTTATACCAGGTTGTAAAGGAGGTAAAACCCGGTGGTCTGGTACAGATTGCCG
ATGGGACTTATAAAGATGTTCAGCTGATTGTCAGCAATTCAGGAAAATCTGGTTTGCCCATCACTA
TTAAAGCCCTGAACCCGGGTAAGGTTTTTTTTACCGGAGATGCTAAAGTAGAGCTGAGGGGCGAGC
ACCTGATACTGGAAGGCATCTGGTTTAAAGACGGGAACAGAGCTATTCAGGCATGGAAATCACATG
GACCCGGATTGGTGGCTATATATGGTAGCTATAACCGCATTACCGCATGTGTATTTGATTGTTTTG
ATGAAGCCAATTCTGCTTACATTACTACTTCGCTTACCGAAGACGGAAAGGTACCTCAACATTGCC
GCATAGACCATTGCAGTTTTACCGATAAGATCACTTTTGACCAGGTAATTAACCTGAACAATACAG
CCAGAGCTATTAAAGACGGTTCGGTGGGAGGACCGGCGATGTACCATCGTGTTGATCACTGTTTTT
TTTCCAATCCGCAAAAACCGGGTAATGCCGGAGGGGGAATCAGGATTGGCTATTACCGTAATGATA
TAGGCCGTTGTCTGGTAGACTCTAACCTGTTTATGCGTCAGGATTCGGAAGCAGAGATCATCACCA
GCAAATCGCAGGAAAATGTTTATTATGGTAATACTTACCTGAATTGCCAGGGCACCATGAACTTTC
GTCACGGTGATCATCAGGTGGCCATTAACAATTTTTATATAGGCAATGACCAGCGATTTGGATACG
GGGGAATGTTTGTTTGGGGAAGCAGGCATGTCATAGCCTGTAATTATTTTGAGCTGTCCGAAACCA
TAAAGTCGAGGGGGAACGCCGCATTGTATTTAAACCCCGGTGCTATGGCTTCGGAGCATGCTCTTG
CTTTCGATATGTTGATAGCCAACAACGCTTTCATCAATGTAAATGGGTATGCCATCCATTTTAATC
CATTGGATGAGCGCAGAAAAGAATATTGTGCGGCCAATAGGCTTAAGTTCGAAACCCCGCACCAGC
TAATGTTAAAAGGCAATCTCTTCTTTAAGGATAAACCTTATGTTTACCCATTTTTTAAAGATGATT
ATTTTATAGCAGGGAAAAATAGCTGGACTGGTAATGTAGCCTTAGGTGTGGAAAAGGGAATCCCTG
TTAACATTTCGGCCAATAGGTCTGCCTATAAGCCGGTAAAAATTAAAGATATCCAGCCCATAGAAG
GAATCGCTCTTGATCTCAATGCGCTGATCAGCAAAGGCATTACAGGAAAGCCCCTTAGCTGGGATG
AAGTAAGGCCCTACTGGTTAAAAGAAATGCCCGGGACGTATGCTTTAACGGCCAGGCTTTCTGCAG
Atagggctgcaaagtttaaagccgtaattaaaagaaataaagagcactga

FIG. 13

TTTACCATTTTGCACAAAATAACCCATTAGCAGACTTCTCATCAGATAAAAACTCAATACTAACGT
TATCTGATAAACGTAGCATTATGGGAAACCAATCTCTTTTATGGAAATGGAAAGGTGGTAGTAGCT
TTACTTTACATAAAAAACTGATTGTCCCCACCGATAAAGAAGCATCTAAAGCATGGGGACGCTCAT
CTACCCCCGTTTTCTCATTTTGGCTTTACAATGAAAAACCGATTGATGGTTATCTTACTATCGATT
TCGGAGAAAAACTCATTTCAACCAGTGAGGCTCAGGCAGGCTTTAAAGTAAAATTAGATTTCACTG
GCTGGCGTGCTGTGGGAGTCTCTTTAAATAACGATCTTGAAAATCGAGAGATGACCTTAAATGCAA
CCAATACCTCCTCTGATGGTACTCAAGACAGCATTGGGCGTTCTTTAGGTGCTAAAGTCGATAGTA
TTCGTTTTAAAGCGCCTTCTAATGTGAGTCAGGGTGAAATCTATATCGACCGTATTATGTTTTCTG
TCGATGATGCTCGCTACCAATGGTCTGATTATCAAGTAAAAACTCGCTTATCAGAACCTGAAATTC
AATTTCACAACGTAAAGCCACAACTACCTGTAACACCTGAAAATTTAGCGGCCATTGATCTTATTC
GCCAACGTCTAATTAATGAATTTGTCGGAGGTGAAAAAGAGACAAACCTCGCATTAGAAGAGAATA
TCAGCAAATTAAAAAGTGATTTCGATGCTCTTAATATTCACACTTTAGCAAATGGTGGAACGCAAG
GCAGACATCTGATCACTGATAAACAAATCATTATTTATCAACCAGAGAATCTTAACTCCCAAGATA
AACAGCTATTTGATAATTATGTTATTTTAGGTAATTACACGACATTAATGTTTAATATTAGCCGTG
CTTATGTGCTGGAAAAAGATCCCACACAAAAGGCGCAACTAAAGCAGATGTACTTATTAATGACAA
AGCATTTATTAGATCAAGGCTTTGTTAAAGGGAGTGCTTTAGTGACAACCCATCACTGGGGATACA
GTTCTCGTTGGTGGTATATTTCCACGTTATTAATGTCTGATGCACTAAAAGAAGCGAACCTACAAA
CTCAAGTTTATGATTCATTACTGTGGTATTCACGTGAGTTTAAAAGTAGTTTTGATATGAAAGTAA
GTGCTGATAGCTCTGATCTAGATTATTTCAATACCTTATCTCGCCAACATTTAGCCTTATTATTAC
TAGAGCCTGATGATCAAAAGCGTATCAACTTAGTTAATACTTTCAGCCATTATATCACTGGCGCAT
TAACGCAAGTGCCACCGGGTGGTAAAGATGGTTTACGCCCTGATGGTACAGCATGGCGACATGAAG
GCAACTATCCGGGCTACTCTTTCCCAGCCTTTAAAAATGCCTCTCAGCTTATTTATTTATTACGCG
ATACACCATTTTCAGTGGGTGAAAGTGGTTGGAATAACCTGAAAAAAGCGATGGTTTCAGCGTGGA
TCTACAGTAATCCAGAAGTTGGATTACCGCTTGCAGGAAGACACCCTTTTAACTCACCTTCGTTAA
AATCAGTCGCTCAAGGCTATTACTGGCTTGCCATGTCTGCAAAATCATCGCCTGATAAAACACTTG
CATCTATTTATCTTGCGATTAGTGATAAAACACAAAATGAATCAACTGCTATTTTTGGAGAAACTA
TTACACCAGCGTCTTTACCTCAAGGTTTCTATGCCTTTAATGGCGGTGCTTTTGGTATTCATCGTT
GGCAAGATAAAATGGTGACACTGAAAGCTTATAACACCAAAGTTTGGTCATCTGAAATTTATAACA
AAGATAACCGTTATGGCCGTTACCAAAGTCATGGTGTCGCTCAAATAGTGAGTAATGGCTCGCAGC
TTTCACAGGGCTATCAGCAAGAAGGTTGGGATTGGAATAGAATGCAAGGGGCAACCACTATTCACC
TTCCTCTTAAAGACTTAGACAGTCCTAAACCTCATACCTTAATGCAACGTGGAGAGCGAGGATTTA
GCGGAACATCATCCCTTGAAGGTCAATATGGCATGATGGCATTCGATCTTATTTATCCCGCCAATC
TTGAGCGTTTTGATCCTAATTTCACTGCGAAAAAGAGTGTATTAGCCGCTGATAATCACTTAATTT
TTATTGGTAGCAATATAAATAGTAGTGATAAAAATAAAAATGTTGAAACGACCTTATTCCAACATG
CCATTACTCCAACATTAAATACCCTTTGGATTAATGGACAAAAGATAGAAAACATGCCTTATCAAA
CAACACTTCAACAAGGTGATTGGTTAATTGATAGCAATGGCAATGGTTACTTAATTACTCAAGCAG
AAAAAGTAAATGTAAGTCGCCAACATCAGGTTTCAGCGGAAAATAAAAATCGCCAACCGACAGAAG
GAAACTTTAGCTCGGCATGGATCGATCACAGCACTCGCCCCAAAGATGCCAGTTATGAGTATATGG
TCTTTTTAGATGCGACACCTGAAAAAATGGGAGAGATGGCACAAAAATTCCGTGAAAATAATGGGT
TATATCAGGTTCTTCGTAAGGATAAAGACGTTCATATTATTCTCGATAAACTCAGCAATGTAACGG
GATATGCCCTTTATCAGCCAGCATCAATTGAAGACAAATGGATCAAAAAGGTTAATAAACCTGCAA
TTGTGATGACTCATCGACAAAAAGACACTCTTATTGTCAGTGCAGTTACACCTGATTTAAATATGA
CTCGCCAAAAAGCAGCAACTCCTGTCACCATCAATGTCACGATTAATGGCAAATGGCAATCTGCTG
ATAAAAATAGTGAAGTGAAATATCAGGTTTCTGGTGATAACACTGAACTGACGTTTACGAGTTACT
TTGGTATTCCACAAGAAATCAAACTCTCGCCACTCCCTTGAGAATTCCGCGGCCGCAGGCCTCTAG
AGTCGACCTGCAGTAATCGTACAGGGTAGTACAAATAAAAAAGGCACGTCAG